Differential Gene Expression in Ginny Cattleya Orchids due to Infection with the Odontoglossum Ringspot Virus: The Culmination of a Year's Work

An Honors Project (Honors 499)

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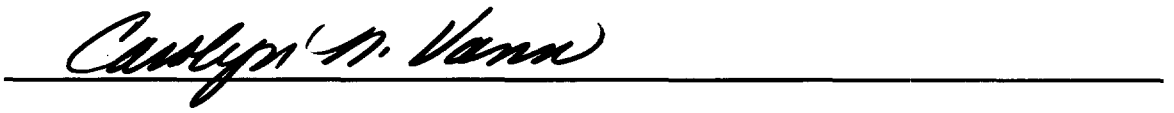

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I would also like to extend thanks to my family for their constant supply of support and encouragement, without which I could never have realized my potential or my dreams.

ABSTRACT

The BSU Wheeler Orchid collection is a reservoir of rare species and one of the most varied species collections in the world. As a species bank, it acts as a clearing house for information and provides plant tissue for orchid collections worldwide. It is important to maintain such an important collection in healthy conditions, however plants are subjected to pathogens resulting from crowding and introduction of new plants that may carry disease. A better understanding of orchid-pathogen relationships is necessary to ensure survival of these endangered species. Thus, understanding orchid-virus interactions at the molecular level may perhaps lead to new strategies for the introduction of specific regulatory genes whose expression may provide preventive protection. In prior research, an orchid was infected with the Odontoglossum Ringspot Virus (ORSV) in hopes of inducing a plant defense response known to prevent viral spread. Molecular subtraction was performed on mRNAs isolated before and after infection to obtain specific rare mRNAs expressed in response to ORSV challenge. The rare mRNA fragments were reverse transcribed and cloned into a vector molecule. The clones were then amplified by PCR, and transformed into *Escherichia coli* from which a differentially expressed cDNA library was formed. DNA miniprep isolations were done to ascertain the diversity and the size of the cloned fragments. Some clones from this library have been sequenced and have yielded homology to certain universal viral genes found in plants. In the near future, the timing and expression of these virally activated genes resulting from pathogenic attack will be pinpointed via Northern analysis of induced mRNA obtained from the same orchid plant.

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INTRODUCTION

Interactions between pathogens and plants have had drastic results on the evolvement of civilization since humans began to rely extensively on cultivated crops for food. In the past, plant disease epidemics have produced disastrous famines and major social change. The effects have been especially harmful in circumstances such as the Irish potato famine in the 1840s, in which a single crop was the main food source. In addition, pathogenic interactions with plants can also affect the health of humans and livestock. One example of this is ergot poisoning, which is caused by the fungus *Claviceps purpurea*, which contaminates rye flour. As a result of the devastating effects of pathogen-plant interactions, further advancements in plant disease resistance are of great interest.

In the past, the incorporation of disease resistance genes in commercially acceptable samples has been the most desirable and effective strategy of plant resistance. Only recent advances in molecular biology have allowed study of the intricacies of these interactions at the cell and molecular level. Many of these advancements have led to the finding that defense mechanisms discovered in plants entail constitutive and induced structural barriers and inhibitory chemical compounds .

Generated responses following pathogenic attack have been discovered to be activated in many ways. Intracellular activation has been found to employ signaling molecules believed to be similar to those that operate in animals. Nevertheless, in spite of the identification of several potential signal molecules, this type of communication in plants is very poorly understood.

Orchids, as a species, have been studied very little in the past. Orchids, in their natural habitat, are near extinction, therefore cultivation in greenhouses have become necessary. Despite this protective measure, however, the close proximity has yielded an effective way for a deadly pathogen to travel and wreak its havoc onto healthy specimens. Therefore, further understanding of the orchid defense process and the genes involved will lead to more improved strategies of cultivation and even resistance.

In previous research conducted by Master's thesis student Heather Shuck, an orchid of the *Ginny Cattleya* species was inoculated with the Odontoglossum Ringspot Virus and tissue was collected at different time points following infection. After collection, Heather isolated mRNA from the orchid and via molecular subtractive hybridization, the rare differentially expressed virally induced mRNA transcripts were copied into complementary DNA (cDNA). The purpose of this research was to clone these cDNAs into plasmid vectors, transform *Escheria coli (E.coli)*, and select transformants to construct a cDNA subtractive library. Subsequent research resulted in the screening of this library via mini-prep DNA isolations in order to confirm the colonies represented did indeed contain the vector and inserted genes. While some of these clones were being sequenced by Heather, mRNA was isolated from leaves at various time points following infection to permit future Northern Analysis of the timing and duration of gene induction. With this information, maybe a better comprehension of plant-pathogen interactions in orchids can be obtained and as a result of this new information, perhaps more effective strategies can be developed which will help protect this endangered species.

REVIEW OF LITERATURE

Odontoglossum Ringspot Virus

Odontoglossum ringspot virus (ORSV) was first described as a pathogen of Odontoglossum grande (Jensen et al., 1951). This pathogen has occasionally been attributed as an orchid strain of the tobacco mosaic virus (TMV) and is known to infect various commercially important orchid genera worldwide (Van Regenmortel, M.H.V., 1986).

In 1971, the Plant Virus Subcommittee of the International Committee on Nomenclature of Viruses classified ORSV along with the tobacco mosaic virus, the tomato mosaic virus, and the cucumber green mottle virus as the tobamovirus group (Harrison, B.D., et al. 1971). These viruses were grouped together because of the common characteristics they share. For example, tobamoviruses contain 5% single-stranded RNA of molecular weight $2X10^6$. Furthermore, they have a sedimentation coefficient of 190S,contain straight tubular particles, protein subunits with a molecular weight of 17,500, and infective particles that are 300 nm in length. Structural characteristics of ORSV are typical of tobamoviruses. In purified and leaf preparations, normal length measurements of ORSV particles were found to be from 280 to 325 nm while diameters ranged from 18 to 27 nm. In negatively stained preparations, these particles exist in distinct channels (Izadpanah, K., et al., 1968) while the coat appears to be composed of stacked disks (Corbet, M.K., 1967).

Analytical centrifugation studies performed have revealed purified ORSV preparations exhibiting a broad peak at 119S and a sharp peak at 211.6. This differs from the single peak observed in similarly treated preparations of TMV. The two peaks correspond to ORSV particles separated into top and middle zones during sucrose density-gradient centrifugation. The top zone contained non-infectious fragmented virions while intact infectious particles 300 nm in length were discovered in the middle and bottom zones. The bottom zone also contained aggregated particles greater than 350 nm in length. Sequence analysis and equilibrium centrifugation performed on ORSV capsids reveal molecular weights of 17598, and 17,300. The capsid subunit of ORSV was

found to contain 157 amino acid residues compared to the 158 found for TMV. (Van Regenmortel, M.H.V., et al. 1986. 234-235).

The distribution of ORSV in commercial orchids is widespread. ORSV was originally thought to spread in native forests because of the detection in 1974 of the virus in *Cattleya* plants imported into the United States from South America. Although this hypothesis could not be confirmed in wild orchids, ORSV was detected in cultivated *Cattleya* and other genus accessions (Van Regenmortel, M.H.V., et al. 1986. pg. 234-235). This suggests that ORSV is a greenhouse problem due in large part to the close proximity in which they are kept.

The stability of ORSV allows it to be spread from orchid to orchid via the use of contaminated tools, contaminated pots, sphagum, and water. The use of sanitary practices in growing orchids is strongly recommended. Orchids infected with ORSV should be burned to prevent viral spread. If the infected orchids are kept, they should be removed from any green house containing healthy orchids (Van Regenmortel, M.H.V pg. 239).

Differences in the range of plant species that TMV and ORSV systemically infect supplement the differences that exist in structure between the two. While both viruses have the capacity to systemically infect *Nicotiana benthamiana* similarly, disparity exists in the ability of each to infect *Nicotiana tabacum* (tobacco). ORSV is confined to the inoculated leaves of *N. tabacum*, however TMV induces a swift systemic infection (Hilf, M.E., et al. 1993). In addition, a strain of TMV which induces bright yellow mosaic symptoms on Nicotiana glauca exhibited no little or no relationship to ORSV (Randles et al., 1964).

In the past, the ORSV has also been referred to as a tobacco mosaic virus strain O in orchids (TMV-O). In order to determine whether these two were really the same virus, a previous graduate student, Audra Carroll, whose work focused on conferring restistance to ORSV in orchids, sequenced the coat protein gene of TMV-O and compared it to the previously reported ORSV sequences. Carroll found that the sequenced TMV-O yielded homology to 3 ORSV sequences with insignificant amino acid differences. The amino acid sequences differences were deemed insignificant because viruses are highly mutagenic. Therefore, it was concluded that

ORSV exists as several species-specific strains that are similar or analogous to TMV-O. On this conclusion, it was determined that TMV-O is one strain of ORSV and the use of the name TMV-O only generates confusion within the tobamovirus group (Carroll, unpublished data).

Active Defense Mechanisms

When sensing an invading pathogen, plants utilize many defense mechanisms in an attempt to restrict pathogen growth and eventually eradicate it (Wojtaszek, P. 1997). The most influential factors governing the outcome of plant-pathogen interactions are the general defense reactions such as cell wall reinforcement, accumulation of antimicrobial compounds, etc, and temporal and spatial regulation (van de Rhee, M.D.. et al, 1993). The events that take place during pathogenic attack are the same regardless of whether the interaction between host and pathogen are compatible or incompatible. The invading pathogen must first penetrate extensive protective layers of the host. This can occur in a variety of ways, such as natural openings, direct penetration, or wounds (Lucas, John A. 1998 pg. 91).

After penetration, events take a different course as host resistance and compatibility must be taken into account. Cell wall alterations are the first observable changes that take place during infection. In plant-fungus interactions, thickening and modification of the cell wall has been observed. Studies show that these new wall deposits are composed of heterogenous papillae form beneath the penetration peg. This papillae is suggested to block fungal penetration of hosts (Byals, C.J., and Orlandi, E.W. 1990).

In addition to papillae formation, callose deposition is associated with sites of pathogen-host incompatibility. These depositions also occur when plants are wounded or threatened by pathogen elicited determinants. Blockage of plasmodemata with callose is an essential component of the defense response that impedes cell-to-cell movement of viruses (Hammond-Kosack, K.E., Jones, J.D.G. 1996).

Hydroxyproline-rich glycoproteins (HRGPs) are also a key component in the fortification of the cell wall. It is thought that HRGPs are a catalyst with lignin polymerization, perhaps a late

defense response to incompatible pathogenic attack, and a companion to the early defense response of the oxidative burst (Hammond-Kosack, K.E., Jones, J.D.G.).

Signal Transduction

If a plant species is immune to a particular pathogen, that virus is termed nonpathogenic. If systemic disease is caused after viral infection, the infecting virus is considered pathogenic. If the particular plant species contains a gene that renders it immune or resistant to a particular gene, the virus is avirulent. If a particular strain does, in fact, cause a systemic infection, that virus is considered virulent.

In what is now called the gene-for-gene model of resistance, a specific resistance gene (R) corresponds to a particular avirulent (*avr*) gene located in the pathogen. *R* genes, which are dominant, act as receptors on the host cell membrane while the avr gene encodes a signal molecule that will bind to the receptor. After the pathogen has successfully penetrated the host cell wall, a complexity of signal transduction pathways occurs in order to stimulate and oversee plant defense responses. *R* proteins are imagined to act as receptors that detect microbial avirulent-dependent signals and initiate downstream signaling. An alternative pathway is also suggested in which an *avr* signal recognition involves a different protein with the *R* protein acting as a very early rate-limiting step in the signal transduction cascade or as a point of cross-talk between different signal transduction pathways. The examination of purified *C.fulvum avr*-encoded peptide elicitors was able to establish a temporary signaling of events that occur in tomato leaves during *R* gene-dependent reactions. These results show that *R* gene-dependent signaling systems can be both kinetically and qualitatively different from one another but conform to the pathways shown in Figure 1 (Dangl, J.L. et al. 1996).

Despite uncertainty that exists concerning the pathway used, it is clear that an elicitor must be used in order to activate plant defense. An elicitor, which may be a peptide, protein, or oligosaccharide, is a product of a pathogen *avr* gene that is recognized by the *R* gene of the host to induce a defense response (Lucas, J. A. 1998).

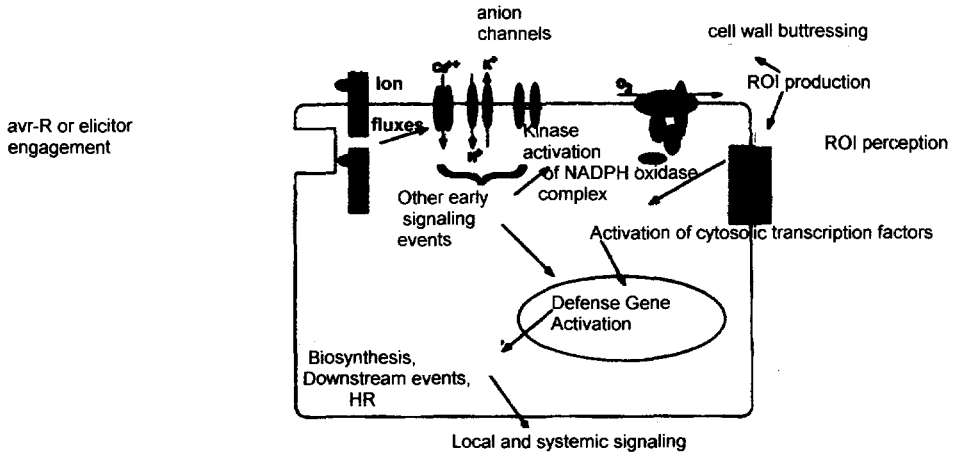

Figure 1. Early Signaling Events in Activation of Cell Death and Disease Resistance. Extacellular and intracellular perception of Avr-dependent or elicitor signals by R gene prducts results in Ca2+ ion infux,; K+/H+ ion exchange leading to ROI production. Other early signals can precede the oxidative burst and can include activation of kinases, phosphatases, phosop peroxidation, and cytoplasmic rearrangement. (Dangle et al. 1996. pg1800).

Downstream from pathogenic recognition, preexisting protein kinases, phosphatases, and G proteins are then activated. A study showing that a protein kinase, Pti, interacts with the tomato Pto protein, implicates the occurrence of a protein cascade in R protein action (Zhou, J., et al. 1995). Other induced events that occur are modifications in Ca^+ concentration induced by reactive oxygen species (ROS), ion fluxes which lead to H^+/K^+ exchange that results in acidification of the intracellular environment, and increased phytoalexin biosynthesis. These events play critical roles in the hypersensitive defense response and will be discussed in more detail in the Hypersensitive Response section.

Modifications to the ratio of proteins bound to GTP or GDP is another event induced when the host plant is attacked. GTP-binding proteins, otherwise known as G proteins, regulate a variety of cellular proceedings. Among these are development, differentiation, and intracellular transportation. G proteins are capable of binding GTP which is hydrolyzed to GTP via GTPase activity of the proteins. This metamorphosis results in the activation of a GDP bound complex which can regulate the basic signaling pathways of the organism. Of the four major groups of G proteins, only the membrane-bound trimeric G proteins and the small GTP-binding proteins are believed to be involved in signal transduction (Sano, H., Ohashi,Y. 1995).

R genes

R genes have been recognized as key components in plant defense for over a century. As a result, they have been extensively studied in various plant species for a number of years. The first *R* gene to be clone was *Hm1* from maize, which conferred resistance to *Cochliobolus coarbonum.* The second *R* gene to be characterized *was Pto* from tomato, which induced resistance to various strains of *Pseudomonas syringae*. In addition to these two, *Xa21* gene from rice as well as *RPS2* and *RPP5* from Arabidopsis have been cloned. In tobacco, the *R* gene cloned was the temperature sensitive dominant *N* gene (Figure 2). The *N* gene encodes a nucleotide binding site (NBS)/LRR that includes N-terminal domain similar implicated in protein-

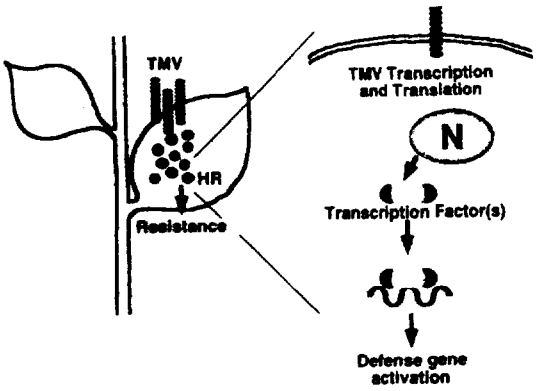

Figure 2. Proposed Mechanism for N Protein-mediated Signal Transduction in Response to TMV infection (Whitham, S. et al. 1994. pg 1111).

protein interactions resembling interleukin 1 receptor-like proteins in mammals. (Whitham, S., et al. 1994). This temperature sensitivity has allowed for study of many defense responses associated with the N gene. For example, it is known that if an N-gene expressing tobacco plant is inoculated with TMV, then kept at 30 degrees Celsius, a systemic infection will result. If the plant is then moved to a 20 degree Celsius environment, incompatibility is imposed, and the hypersensitive response is induced. At this point, since the virus has been systemically spread, the inducement of the hypersensitive response is lethal to the plant (Lucas, J.A. 1998.)

Tobacco plants encoding for the *N* gene are said to be resistant and therefore able to induce hypersensitive lesions within 48 hours after infection. Those tobacco plants containing the recessive gene (*nn*) allow TMV to spread systemically. As a result of this systemic infection, these plants were termed TMV-sensitive and designated as SR 1 *nn*. By transforming the SR 1 *nn* plants so that the *N* gene was introduced into its genome, it was discovered that TMV resistance could indeed be conferred and the hypersensitive response could be induced (figure 2). This suggested that the *N* gene was the main determinant for hypersensitive resistance. In addition, defense responses induced during the HR such as phytoalexin synthesis, lignin deposition, and chitinase production, were found to be produced only in plants containing the *N* gene (Whitham, S., et al. 1994).

The Hypersensitive Response

A particularly interesting response is the activation of the hypersensitive response (HR), which serves not only as a dynamic role of the host early in pathogen attack, but also as a way to confer a high degree of resistance to the host. The HR appears as necrotic lesions resulting from the death of a small number of cells at the infection site due to incompatible interactions between the host plant *R* genes and *avr* genes. The necrotic lesions, which usually appear within a few hours of pathogenic attack, serve to deprive the pathogen of access to further nutrients, thus

starving it, restricting its growth, and physically walling it in (Hammond-Koscack K.E., Jones, J. 1996). The number of cells that are affected by the HR is only a small percentage of the total plant, thus this response contributes to the survival of the plant undergoing pathogenic attack. In effect, the plant is sacrificing several cells so the rest can survive.

The trigger of HR is thought to be the accumulation of reactive oxygen intermediates (ROI) in response to microbial elicitors induced by avirulant pathogens. This accumulation has been termed as the oxidative burst (Figure 3) (Tenhake, R., et al.,1995)

It was reported that incompatible reactions between tobacco and the tobacco mosaic virus results in the formation of a superoxide anion, $O_2^{\cdot-}$ via a NADPH oxidase (Levine, A., et al. 1994). The superoxide anion, which cannot cross cell membranes and is only moderately reactive. However, it rapidly undergoes dismutation via superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2), which is more noxious and can cross cell membranes because of a lack of unpaired electrons. This compound is produced by cell wall peroxidases. Many roles found for H_2O_2 include toxicity to pathogens, reinforcement of plant cell walls via oxidative crosslinking, formation of ligin polymer precursors, and an increased production of benzoic acid-2 hydroxylase enzyme activity. This enzyme leads to synthesis of salicylic acid, which acts as signaling molecule (Leon, J., et al. 1995).

In addition to conversion to H_2O_2, the superoxide anion can be converted to the hydroxyl radical, $HO_2^{\cdot}$, by protonation. This radical degrades membranes and produces a wide variety of lipid peroxide signal molecules (Figure 4A).

In the presence of the Fe^{2+}, H_2O_2 can undergo the Fenton reaction, which gives rise to the very toxic hydroxyl radical ($OH^{\cdot}$) which induces lipid peroxidation (Figure 4B). If H_2O_2 enters the cytoplasm of the cell and reaches the plant nucleus in sufficient concentration, it could react with metal ions that exist intracellularly to produce $OH^{\cdot}$, which is known to fragment DNA.

In addition to the cell death that occurs at the site of infection, there are many studies that show that the HR is supplemented by biochemical changes that occurs both at the site of inoculation and at distant sites on the plant. As mentioned previously, at the site of inoculation

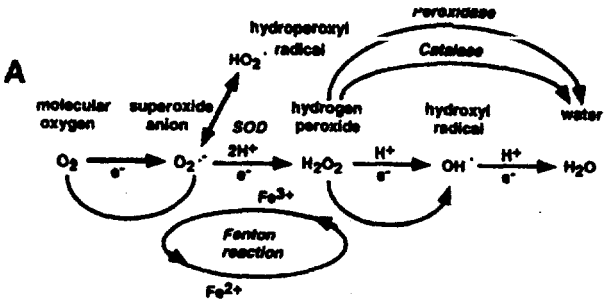

Figure 3. The General Scheme of the Oxidative Burst (Hammond-Kosack et al 1996 pg 1776)

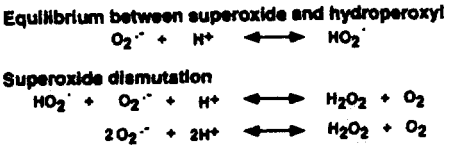

Figure 4A. Chemical Equations showing key reactions involving the conversion of the superoxide ion to hydrogen peroxide via superoxide dismutase (Hammond-Kosack et al. 1996. pg1776).

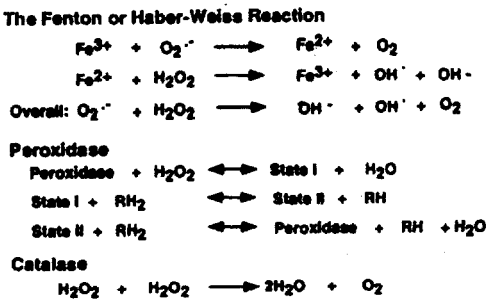

Figure 4B. The General Mechanism of the Fenton or Haber-Weiss Reaction (Hammond-Kosack et al. 1996. pg 1776).

the HR is connected with an inducement of certain defense-related genes, an influx of Ca^{2+} , and a K+//H+ response (Atkinson, M.M., et al. 1990). The breakdown of membranes and the cellular chaos that supplements necrosis may trigger metabolic changes in living cells which consequently prevents viral spread.

Plant cells attacked by a pathogen encoding an *avr* gene have been shown to produce elicitors that induce reactive oxygen intermediate production within 5 minutes. This ROI production requires an influx of Ca^{2+} at the plasma membrane. This suggests that Ca^{2+} influx is induced within hours after inoculation and that membrane transport is an important factor in the HR. Studies have shown that Ca^{2+} channels are blocked by La^{3+}, Cd^{2+}, and Co^{2+}. Plants treated with these calcium channel blockers inhibited the K^+/H^+ response as well. The blockage by these cations implies that the Ca^{2+} influx is regulated by ion channels and is required for the K^+/H^+ response. In addition, the cell death that occurs after Ca^{2+} blockage indicates that sustenance of the HR requires a consistent Ca^{2+} influx, which supports the second messenger role that Ca^{2+} plays during the HR (Atkinson, M.M.,et al. 1990).

The K^+/H^+ response induced by the calcium ion influx has a devastating effect on cellular morphology after viral infection. Within 5 hours after infection, this exchange resulted in 35% loss of total K^+ and acidification of the internal cellular environment. The resulting consequences are catastrophic. The low concentration of K^+ compromises the stimulation of many enzymes and the energy conservation across membranes. In addition, the reduced K^+ concentration coupled with a more acidic cellular environment severely inhibit respiration, RNA synthesis, and ATP levels in hypersensitive cells. The net H^+ influx reduces the ATPase-regulated H^+ influx across the plasma membrane, thus inhibiting active transport and actively stimulating ATPase and respiration. This accounts for the respiratory stimulation which occurs early in the HR (Atkinson, M.M., et al. 1985).

PR proteins

In TMV-tobacco studies, it was noticed that after inoculation with TMV there was an accumulation of many soluble host cell proteins within the host cell. These proteins were differentially expressed in response to incompatible pathogen-plant interactions (Bol, J.F., et al. 1990. In tobacco expressing the *N* resistance gene, 14 acidic PR proteins are induced. These proteins include those that are found intracellularly and extracellularly in infected plant tissue. Their appearance is easily authenticated by gel electrophoresis of inoculated tissue extracts (Figure 5) (Lucas, J.A., 1998).

PR proteins have been found to be elicited by salicylic acid (SA), a key ingredient for the development of systemic acquired resistance (SAR). SAR is usually preceeded by the hypersensitive response and results in the development of resistance of the entire plant, to subsequent infection (Lee, H.I., et al. 1995).

PR proteins, because they are only found post-inoculation, have some very important characteristics. For instance, PR proteins are induced by a series of corresponding mRNAs in the infected leaf. PR-1 proteins found in tobacco and tomatoes inhibit the growth oomycete pathogens such *Phytophthora iinfestans*. PR-2 and PR-3 activates enzymes against polysaccharides containing B-1,3-glucans or chitin. These functions, thus, suggest that PR proteins play a role in plant defense (Lucas, J.A. 1998).

PR proteins can be induced by various chemical treatments and numerous pathogens, such as bacteria, fungi, and viruses. They can also be viral infections that cause necrosis in plants that do not carry the N gene. Furthermore, proteins that exhibit homology to PR proteins have been found in 20 plant species in monocotyledons and dicotlyedons (Lucas, J.A., 1998).

Programmed Cell Death

Programmed cell death (PCD) is an active process resulting in cell death that plays a critical role in the host plant defense response to pathogens. PCD in the HR response results in

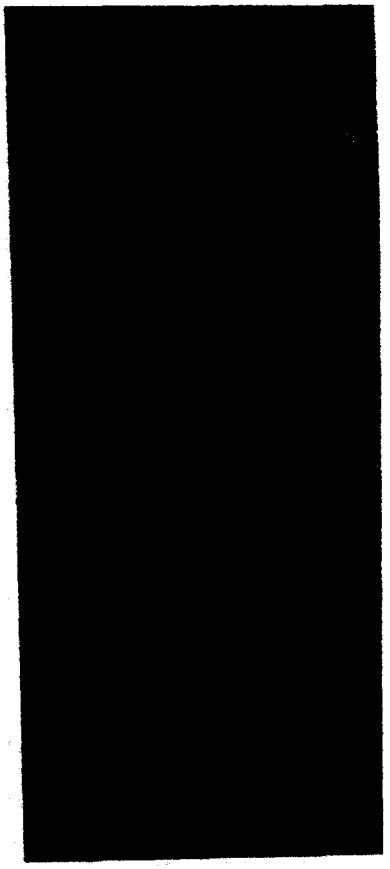

Figure 5. Gel electrophoresis of proteins extracted
from healthy tobacco leaves (H) and leaves forming
local lesions in response to inoculation (I) with the TMV.
The extra bands in I are the appearance of PR prtoeins (Lucas, J.A. 1998, pg. 153).

triggering signal transduction pathways occur. Following the induction phase is an effector phase in which numerous PCD-eliciting stimuli converge into a few stereotypical pathways. It is also during this phase that the cells pass the "point-of-no return." After this, the cell is irrevocably designated to undergo death. The following degradation phase results in the destruction of vital structures and functions that result in the all-out appearance of PCD. Prior to this stage, all external membranes remain unaltered (Kroemer, G., et al. 1995).

In the leading induction phase of PCD, the cell responds to several combinations of signals. Among these are physiological signals, such as the presence of death signals or the lack of survival signals, the presence of contradictory signals, and physical or chemical damage. In actuality, there is no process or event that always triggers the inducement of PCD. A stimulus will only induce PCD in a certain context such as a specific position of the cell cycle , the existence of its regulators such as p34., and the products of proto-oncogenes like *rel* and *c-myc* exist. Despite the numerous ways in which PCD can be elicited, the induction phase, however, does not exhibit any morphological alterations or reduction in cell size (Kroemer, G., et al. 1995).

The subsequent effector phase is characterized by the presence of a "point of no return" which renders the cell its fate. Different physiological stimuli converge to form a few customary molecular triggers that determine the fate of the cell. In this stage, a series of proto-oncogenes exist that influence the life and death decision which must be made. Among these are *Bcl-2* family. *BCl-2*, *bcl-XL (ras)*, *bcl-XB (raf)*, *A1*, *Mcl-1*, and *BAG-1* are all proto-oncogenes . From the same family, *bax*, *bcl-XS*, *bad*, and *bak* are onco-suppressor genes that induce PCD. The members of this family have been found to localize in mitochondria and most likely play a role in mitochondrial chatastrophe, depending on their function. In addition, proteases such as Interleukin(IL) 1B converting enzyme (*ICE*) and its corresponding proteinase, *pe(ICE)* may also elicit PCD by producing nuclear DNA fragmentation via the cleavage of poly ADP-ribose polymerase. The *ICE/ced-3* family member, *ICH1* encodes two alternatively spliced mRNA species. Furthermore, *ICH-1S* inhibits the *RAF-1* cell death resulting from serum deprivation.

necrotic lesions, therefore preventing spread of the infection by limiting pathogen growth to the site of inoculation. As a result, HR is often classified as a type of PCD in plants.

PCD in plants has been considered an analogous process to apoptosis in animal cells. This assumption was made due to the appearance of apoptotic bodies that are characteristic of animal cells undergoing apoptosis. In addition, cell death accompanied by endonucleolytically cleaved DNA in PCD is also a trademark of animal apoptosis. Other apoptotic characteristics include membrane blebbing, nuclear condensation and fragmentation, and cytosolic compression. However, these characteristics have yet to be determined as characteristics of plant PCD, thus supporting the idea that programmed cell death and apoptosis are not interchangeable. PCD is considered a cell death that is natural part of the host plant's lifecycle whereas apoptosis is distinguished by cellular and physiological morphology (Greenburg, J.T. 1996).

As previously mentioned, plant PCD is considered an active process because it requires transcriptionally and translationally active host tissue. It was also determined that HR inducing bacteria cannot induce the HR if protein synthesis is blocked in the cell. This coupled with the fact that HR is induced via certain elicitors suggests that PCD in plants is not caused by the pathogen, but from the activation of a cell death pathway encoded by the plants (He, S.H., et al.1993).

Hydrogen peroxide, already deemed a trigger for the HR response, is also considered to be the trigger of HR induced PCD. When inoculation of soybean cells with *Pseudomonas syringae pv. Glycinea* (Psg) carrying *avrA* was performed, a temporary oxidative burst was aroused. Cell death was not detected until after a lag of 2-4 hours, and a definite increase was noted after eight hours (Figure 6). This lag was thought to perhaps be dependent on the dose of H_2O_2. At low doses of H_2O_2 that are not sufficient to induce PCD, protectant genes were found to be stimulated. The cross-linking of the cell wall that results from the oxidative burst in HR provides a more effective means of trapping the pathogen within the infected cell already designated to undergo PCD, further linking HR with PCD as a defense mechanism (Levine, A.,et al. 1994).

Despite the fact that PCD and apoptosis are two distinguishable processes, their methods of action appear to be similar. Both consist of an initial induction phase in which several PCD-

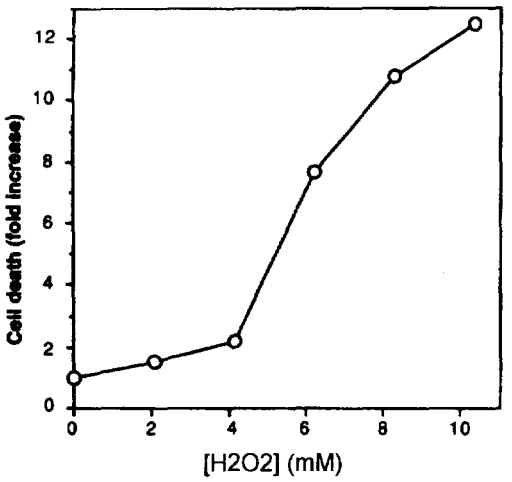

Figure 6. Dose response for Hydrogen Peroxide induction in soybean. Cell death was measur inn specific time frames after the addition of H2O2 (Levine C. et al., 1994, pg 588).

These molecules are also believed to participate in a self-amplifying loop which further enhances PCD and destruction of the cell (Kroemer, G., et al. 1995).

The localization of the *Bcl-2* family in the mitochondria produces many devastating consequences that distinguish the effector phase. Alterations in the mitochondria instigate nuclear modifications in PCD that are evident in the degradation phase. However, before these modifications occur, a reduction in mitochondrial transmembrane potential occurs. This results from an uneven balance of charges between the inner and outer side of the inner mitochondrial membrane. Therefore, the existence of an ion-permeable membrane and a proton pump are required to maintain this potential, which is critical in the foundation of an electrochemical proton gradient and ATP synthesis. If this membrane potential is reduced, transcription and translation in the mitochondria is severely compromised. If the transmembrane potential is low, the cells are designated to undergo pcd. Once this occurs, mitochondrial electron transport is uncoupled from ATP synthesis. This results in the production of $O_2^{\cdot-}$, NADPH consumption, and dispersion of Ca^{2+} between the mitochondria and the cytoplasm. Thus, what occurs here in the effector phase lays the groundwork for the appearance of apoptosis in the degradation phase (Kroemer, G., et al. 1995).

The degradation phase of PCD is typified by several events. These include the increase in internal Ca^{2+}, formation of ROS, ATP deficiency, NAD/NADH depletion, and the inducement of tyrosin kinases and G proteins. The ROS effects, increased Ca^{2+} concentration and G proteins activate endonucleases, repair enzymes, degradation of chromatin and nucleolysis, among others (Figure 7). Successive activation of repair enzymes causes ATP and NAD/NADH deficiency that compromises energy metabolism. This occurs because NADH is consumed by poly (ADP-ribose) polymerase, which is activated by *pe(ICE)*.

The presence of cations, particularly Ca^{2+} has been implicated in all phases of PCD. It acts as a second messenger in the induction phase, as *Bcl-2* regulated cofactor in the effector phase, and as an activator of proteases and nucleases in this last degradation phase. Mittler and Lam examined the induction of nuclease activity during HR induced PCD in order to determine

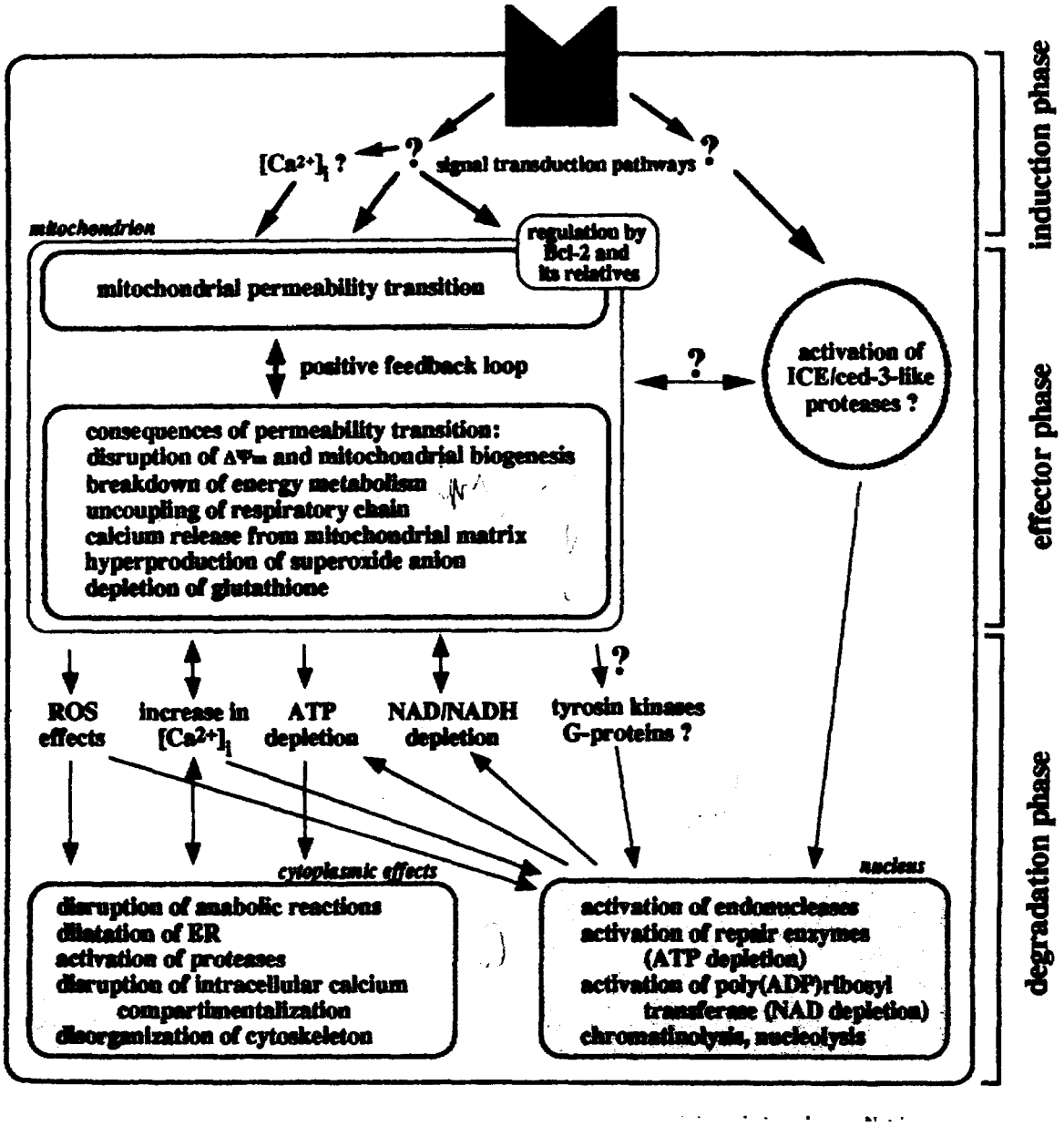

Figure 7. Hypothetical sequence of events accompanying the induction, effector, and degradation phase of programmed cell death (Kroemer, G. et al. 1995 pg. 1285).

whether this correlated with the rise in nuclease activity observed in animal apoptosis. They discovered that TMV infected plants that did not contain the *N* gene, and could not respond with a HR cell death, did not induce nuclease activity, demonstrating the specificity of nuclease activity to HR associated PCD. They were also able to determine that nuclease activity was not associated with general defense mechanisms such as PR proteins and systemic acquired resistance, further demonstrating this activity was associated with connected with programmed cell death (Mittler, R., Lamb, E. 1995).

Mittler and Lam via an in situ assay to detect the presence of 3' OH groups of degraded nuclear DNA, found that nuclei in cells undergoing PCD containing fragmented nDNA (Mittler, R., Lam, E. 1995). Since animal apoptosis is typified by the formation of a DNA ladder, i.e., DNA fragments that are multimers of approximately 180 base pairs, DNA isolated from TMV infected plants undergoing PCD were tested for the formation of DNA ladders. It was discovered via gel electrophoresis that HR induced PCD did not result in the formation of DNA ladders, although regions of fragmented DNA were ascertained. This proves that DNA is not degraded at linker sites between nucleosomes and that DNA degradation occurs via a different pathway, further supporting the assumption that PCD and apoptosis are not interchangeable (Mittler and Lam. 1995. *The Plant Cell*).

Despite the fact that degraded nuclear DNA has been detected in TMV infected plants undergoing HR associated PCD, no internucleosomal fragmentation was ascertained in DNA isolated from these plants. Mittler and Lam did detect the cleavage of chromatin to 50 kb fragments. Therefore they suggested that DNA degradation during TMV dependent PCD involved cleavage of chromatin followed by disorganized degradation, which results in a random pattern of DNA fragmentation. This result accounts for the lack of a DNA ladder in TMV induced PCD.

Other changes that occur during the degradation phase late in apoptosis are changes in the cyostol such as vacoulization, disruption of anabolic reactions such as macromolecule synthesis, destruction of RNA, destruction of the nucleolus, and loss in energy-rich phosphates.

This is similar to apoptosis in animal cells and occurs prior to nuclear modifications, suggesting that perhaps cytoplasmic alterations play an important role in PCD (Kroemer, G. et al. 1995).

Previous Research

In previous research, our laboratory inoculated the Ginny *Cattleya* species of orchids contained in the Wheeler Orchid Collection housed at Christy Woods with the recently discontinued Odontoglossum Ringspot Virus. Graduate student Heather Schuck, currently known as Heather Bullock, performed a technique known as molecular subtractive hybridization to isolate rare differentially expressed RNA from the infected orchid (Schuck, unpublished data).

Subtractive hybridization allows the experimenter to take two populations of mRNA and procure clones of genes that are expressed in one population and not the other. In this technique, both populations of mRNA were converted into cDNA. The cDNA containing the desired transcripts was hybridized with the reference cDNA. The unhybridized cDNA, therefore, contained genes expressed in the desired transcripts, but not in the reference transcripts. Hence, after two rounds of hybridization and two rounds of PCR amplifications, differentially expressed genes induced by pathogenic attack were obtained (CLONTECH PCR-Select cDNA Subtraction Kit User Manual).

MATERIALS AND METHODS

Ligating into pT-Advantage Vector

Ligation into the pT-Advantage vector was performed to obtain clones of the rare, differentially expressed cDNA from the ORSV-inoculated orchid. Ligation was completed with the AdvanTAge PCR Cloning Kit from Clontech (Clontech Laboratories, Palo Alto, California). One tube of pT-Adv vector was centrifuged for every ligation reaction. Three ligation reactions were created with varying volumes of sterile H_20 and freshly amplified PCR product from the subtractive hybridization This was done to determine whether the concentration of PCR product effected the efficiency and success of the reaction. In the first ligation reaction, 2 µl of the pT-Advantage Vector was added to 0.5 µl of TAQ polymerase, 0.5 µl of PCR product, 1 µl of 10 X ligation buffer, 5 µl of sterile H_2O, and 1 µl of T_4 DNA ligase. The second ligation contained 2µl pT-Advantage Vector, 0.5 µl of TAQ polymerase, 1 µl of PCR product, 1 µl of 10 X ligation buffer, 4.5 µl of sterile H_2O, and 1 µl of T_4 DNA ligase. The third reaction contained 2µl pT-Advantage Vector, 0.5 µl of TAQ polymerase, 2 µl of PCR product, 1 µl of 10 X ligation buffer, 4.5 µl of sterile H_2O, and 1 µl of T_4 DNA ligase. All three reactions each had a total volume of 10 µl. After the mixtures were created they were incubated at 14 °C overnight.

Transformation

Transformation of the cDNA clones containing the pT-Adv vector into E. coli bacteria was performed using the AdvanTAge PCR Cloning Kit from Clontech. After the three ligation reations were incubated at 14 °C overnight, they were centrifuged and placed on ice.

On ice, one tube of 0.5 M of B-Mercaptoethanol and one tube of frozen TOP 10F' E.coli competent cells for each transformation was thawed. Two µl of 0.5 M of B-Mercaptoethanol was pipetted into each tube of E.Coli containing bacteria and mixed gently via the pipet tip. Two µl of one ligation reaction was then pipetted into each mixture and also mixed gently with the pipet tip. The tubes were thus incubated on ice for 30 min.

Each tube was then heat shocked in a 42°C water bath and then incubated in ice for two minutes. Two hundred and fifty µl of SOC medium was added to each tube and they were shaken

horizontally at 37°C at 225 rpm in a rotary shaking incubator. After one hour, the tubes containing the transformed cells were placed on ice and then pooled

Five tubes of previously created top agar was boiled for five minutes, then cooled to approximately 50°C for plating. These tubes were then mixed with the pooled competent cell/ligation mixture: 2 tubes with 40 µl o f pooled liquid, 2 with 60 µl of pooled liquid, and 1 with 50 µl of pooled liquid. Five µl of 50 mg/ml of ampicillin and 50 µl of XGAL was added to each top agar/ligation tube and gently swirled. The five mixtures were then spread over five respective Luria Broth/X-GAL plates, allowed to harden, and incubated at 37°C for 36 hours. The plates were then shifted to 4°C for eight hours color development.

Nylon Transfer.

After the plates were shifted to 4°C for eight hours, they were checked in order to determine if the transformation was successful. The transformation was deemed successful if there was the appearance of blue colonies which served as negative controls (i.e. no insert) and white colonies, which contained the pT-Adv vector. Several white colonies were isolated from each plate and spread onto other Luria Broth/X-Gal plates via a toothpick and incubated at 37°C. After the colonies were spread, the toothpick was used to inoculate tubes of Luria Broth containing ampicillin. These tubes would be used to store the cDNA library and to perform DNA isolation screens to further verify the success of the transformation.

After the transferred colonies were incubated for approximately 24 hours and bacterial growth was seen, they were deemed ready for nitrocellulose transfer. One circle of nylon was placed on every plate and oriented with needles. Next, each circle was placed in denaturing solution to denature the DNA on the membrane. After 5 minutes and the membrane was agitated to remove excess DNA from the membrane. The nylon was placed in neutralizing solution for 5 minutes and thereupon agitated until the colonies were shaken off. The subsequent step involved transferring of the nylon membrane to 2X SSC solution for 30 seconds. Following this, the 5 membranes were blotted with a paper towel and allowed to air dry for one hour. They were then transferred to a vacuum oven and baked at 80°C. After one hour, the membranes were sealed with a microsealer and sealed where they can be stored indefinitely.

Storing of the cDNA library

The inoculated LB/X-Gal tubes from the above procedure were then taken and placed in a rotating incubator overnight to facilitate bacterial growth. After growth was seen, two milliliters of this bacterial-containing solution was mixed with glycerol so that a 15% glycerol stock solution was made. Once this was accomplished, the stock solutions can be stored for a long period of time at -20°C.

DNA Miniprep Isolations

DNA miniprep isolations were conducted in order to ascertain if the transformation was indeed successful and the percentage of colonies isolated that actually contained inserts. The isolations were conducted with products provided by the 5 Prime→3 Prime Insta-Mini-Prep Kit (5 Prime->3 Prime Inc., Boulder, Colorado) and the Bio-Rad Quantum Prep Plasmid Miniprep Kit (BIO-RAD Laboratories, Hercules California).

With the 5 Prime-3 Prime kit, an unopened INSTA-MIN-PREP tube was centrifuged at 12,000 rpm for 30 seconds to pellet the INSTA-MINI-PREP gel. Following this step, 1.5 mL of bacterial culture grown overnight was transferred to a 1.5 mL microcentrifuge tube and centrifuged for 30 seconds at full speed. After centrifugation, the supernatant was aspirated and 100 μl of 2X TE buffer was added to the pellet and the resulting solution was vortexed vigorously to resuspend the bacteria. The entire volume of resuspended bacteria was transferred to the pre-spun INSTA-MINI-PREP Tube. Three hundred microliters of shaken phenol chloroform isoamyl Alcohol was added to added to the pre-spun INSTA-MINI-PREP tube and mixed vigourously in order to release the plasmid DNA. The tube was centrifuged for 30 seconds at full speed. The supernatant was then isolated and mixed with 300 μl of chloroform isoamyl alcohol. The solution was thoroughly mixed and centrifuged for 30 seconds at full speed. The supernatant containing plasmid DNA was recovered and transferred to a new microcentrifuge tube. This DNA was then analyzed for inserts via gel electrophoresis and with comparisons to negative controls (no insert).

With the Bio-Rad Quantum Prep Plasmid Miniprep Kit, 1. 5 ml of a bacterial culture was added to a microcentifuge tube and centrifuged for 30 seconds at 12,000 rpm. All of the resulting

supernatant was aspirated and 200 µl of Cell Resuspension Solution was added to the pellet and vortexed until the cell pellet was completely resuspended. After resuspension, 250 µl of the Cell Lysis Solution was added and the tubes were mixed gently by gently inverting10 times. Two hundred and fifty microliters of Neutralization Solution was added and again the tube was gently inverted 10 times. The cell debris was then pelleted for 5 minutes in a microcentrifuge. In the 5 minutes of centrifugation, a Spin Filter supplied from the kit was inserted into a 2 ml was tube also supplied by the kit. The Quantum Prep matrix was repeatedly shaken and inverted to ensure suspension. After the five centrifugation, the lysate containing the plasmid DNA was transferred to the Spin Filer and 200 µl of the suspended Quantum Matrix was added and mixed by pipetting up and down. Following addition of the Quantum Matrix, the tube was centrifuged at full speed for 30 seconds. After centrifugation, the Spin Filter was removed from the 2 ml tube, the filtrate was discarded and the filter was placed in the same tube. Five hundred microliters of Wash Buffer was added to wash the matrix. The mixture was then centrifuged for 2 minutes to remove residual traces of ethanol and the Spin Filter was removed and the microcetrifuge tube was discarded. The filter was placed in a new 1.5 ml microcentrifuge tube and 100 ul of 2X TE buffer was added to elute the DNA . The tube was then centrifuged for 1 minute at full speed. The resulting decantate contained the plasmid DNA and was analyzed for inserts.

Gel Electrophoresis

Gel electrophoresis was conducted in order to determine whether white colonies isolated after transformation did indeed contain pT-Adv vector inserts. Thus a 1.5 % agarose gel was performed on plasmid DNA isolated using both procedures. This gel was run using 0.45 g of Gibco UltraPure Agarose in 3 ml of 10 X E buffer and 27 ml of millipore H_2O. The DNA analyzed included 6 samples believed to have inserts, one sample known to be a negative control, and one lane reserved for 400 ng. Lambda/Hind III Molecular Weight marker. The lanes containing the DNA contained 15 µl of sample and 3.5 µl of gel loading buffer. The gel was allowed to run for approximately 90 minutes at 50 volts and was subsequently analyzed using the ultraviolet transilluminator. A gel electrophoresis picture of one of these screens is represented in Figure 8.

The lane assignments and their significance will be discussed further in the Results and Discussion.

DNA Sequencing

DNA sequencing of 1 gene shown to have inserts was conducted by Heather Bullock and Audra Carroll at Riley Hospital in Indianapolis, Indiana. Bullock and Carroll used M13 Forward and Reverse Primer supplied by Clontech to conduct their sequencing.

RNA Isolations

After DNA isolations and sequencing was completed, it was determined necessary to isolate RNA from infected tissue of the orchid collected at different times so as determine the timing of expression of any virally induced genes. These isolations were performed using Qiagen Rneasy Plant Mini Kit. RNA was isolated from tissue collected from the opposite leaf of a healthy Ginny Cattleya orchid, at Day 1 of the infection, Day 2, Day 11, and Day 42. In addition, tissue was collected on Mach 29,1999, approximately two years after the initial inoculation. These samples were obtained by simply collecting portions of the leaves on these designated days. Once collected, the inoculated tissue was stored in a -70°C cooler located in Cooper Science.

All glassware and solutions used in the RNA isolation were treated with 0.1% diethyl pyrocarbonate, which is a strong inhibitor of Rnases, enzymes which will degrade any RNA obtained. Glassware was treated with 0.1% DEPC overnight at 37oC and then autoclaved for 15 minutes to remove residual DEPC. Solutions were treated by addition of 0.1 mL of DEPC to 100 mL of desired solution and shaken vigorously to bring the DEPC into solution. All treated solutions were then subjected to autoclaving for 30 minutes to remove the residual DEPC.

In this procedure, 0.2 grams of infected orchid tissue collected at different time points was ground to a fine powder under liquid nitrogen with siliconized DEPC-treated sand with a mortar and pester. The tissue powder was transferred to a 1.5 mL microcentrifuge tube and placed in ice so that the sample did not thaw. Four hundred and fifty microliters of Buffer RLT along with 4.5 µl of B-Mercapatoethanol was added to the tube. A three minute incubation in a 56°C water bath followed in order to disrupt the tissue. The lysate was then transferred to a QIA shredder spin

column placed in a 2 mL collection tube supplied by the kit and cetrifuged at full speed for 2 minutes. The flow-through fraction resulting from this process was transferred to a 1.5 mL collection tube, Following this step, 225 µl of 96% ethanol was added to the lysate and mixed by pipeting. The sample was then transferred to a Rneasy mini spin column placed in a 2-mL collection tube and cetrifuged for 15 seconds at full speed. After centifugation, the flow-through was discarded and the same microcentrifuge tube was reused. Seven hundred microliters of Buffer RW1 was added to the Rneasy column and centrifuged again for 15 seconds. The flow-through was again discarded along with the collection tube. The Rneasy column was then put into a new 2 mL collection tube supplied by the kit and mixed with 500 µl of Buffer RPE onto the Rneasy column. This tube was then centrifuged for 30 seconds and the flow-through was discarded while the microcentrifuge tube was reused. Five hundred microliters of Buffer RPE as added to the reused column followed by centrifugation for 2 minutes at maximum speed to dry the RNeasy membrane. The flow-through and collection tube were discarded while the RNeasy column was transferred to a 1.5 mL collection tube supplied by the kit. Fifty ml of Rnase-free H2O was added to the membrane followed by centrfugation for 1 minute at maximum speed for elution of RNA. The column was then discarded and the RNA was read via the use of a UV spectrophotometer at a wavelength of 260 nm in order to determine the yield obtained.

RESULTS AND DISCUSSION

Transformation into pT-Advantage Vector

The transformation yield five plates of Escheria coli bacteria containing the pT-Advantage vector. The plate designated plate A contained 40 μl of competent cell/ligation reaction, plate B contained 60 μl of this mixture, and plate C was plated with 50 μl. Plate D represented the plate plated with 40 μl and Plate E was the second plate with 60 μl.

Success of the transformation was gathered via the appearance of white and blue colonies, however all plates were varied in their white colony to blue colony ratio. These rations are shown in Table 1. All volumes plated yielded white colonies and blue colonies, a sign that the molecular subtractive hybridization technique worked. Of the transformations performed, the 40 μl volume of competent cell/ligation mixture appeared to give the greatest percentage of white colonies. Those plates accompanied by an asterisk indicate that after a few days, those colonies that originally appeared white, appeared to be completely blue or contained blue centers. This suggests that perhaps the initial ratio of white to blue colonies may not be accurate.

Table 1. Transformations

Plate	Volume of sol. plated	White Colonies	Blue Colonies	Percentage (White colonies/total *100)
A	40 μl	75	2	97.4%
B	60 μl	38	5	88%
C*	50 μl	55	10	84%
D*	40 μl	68	6	91.89%
E*	60 μl	62	15	80.51%

CDNA library storage

From the plates containing the transformed E. coli bacteria, 208 colonies were stored in 15% glycerol stocks and are currently being kept in a -20°C freezer. The percentages of those containing inserts were determined via DNA miniprep screens.

DNA Miniprep Isolations

As previously mentioned, DNA miniprep isolations were conducted via the aid of Bio-Rad's Quantum Prep Plasmid Miniprep Kit. This was done with the goal of obtaining a percentage of clones containing inserts several DNA screens were completed.

After it was deemed that the transformation was successful, the colonies were blotted onto a nylon membrane, stored into a subtractive cDNA library, and screened. The presence of an insert was detected via the use of gel electrophoresis. Those samples containing the inserts were shown to migrate slower than those with no inserts. Thus, those bands are located higher than those with no inserts. Figure 8, represents one of the screens performed using gel electrophoresis. Lane 1 represents 400 μg of Lamda/Hind III. Lane 2 represents colony 42a. Colony 42a represents the 42 colony isolated and stored from plate A containing 40 μg/ml of the ligation/competent cell mixture. Lane 3 represents the twenty-first colony isolated from plate A. Lane 4 contains a blue colony which served as our negative control. Lane 6 contained the eighteenth colony isolated from plate A. Lane 7 contained the thirty-fourth colony isolated from plate A. Lanes 5 and 8 were left blank. By examination, it is apparent that lanes 3, 4, and 7 migrate a greater distance than lanes 2 and 6. This result is expected with the negative control in lane four because it contains no pT-Advantage vector insert, therefore it is lighter. Since lighter samples migrate faster than heavier samples in gel electrophoresis, the negative control was expected to migrate further than any sample containing the vector. Lanes 3 and 7 which contained isolated colonies migrated at a rate comparable to the negative control. This implies that these samples, though they may have appeared white after transformation, were not successfully transformed therefore, they do not contain the pT-Advantage vector. Samples 42a and 18a in Lanes 2 and 6, however, did not migrate as far as the negative control. This result suggests these samples are heavier, therefore they contain the 3.9 kilobase pair vector. Thus, these sample contain differentially expressed genes.

Thirty-six screens were performed using both kits on various white bacterial colonies isolated from all five plates. Of the thirty-eight screens performed, twenty showed inserts, resulting in at least a 55.56% successful transformation rate

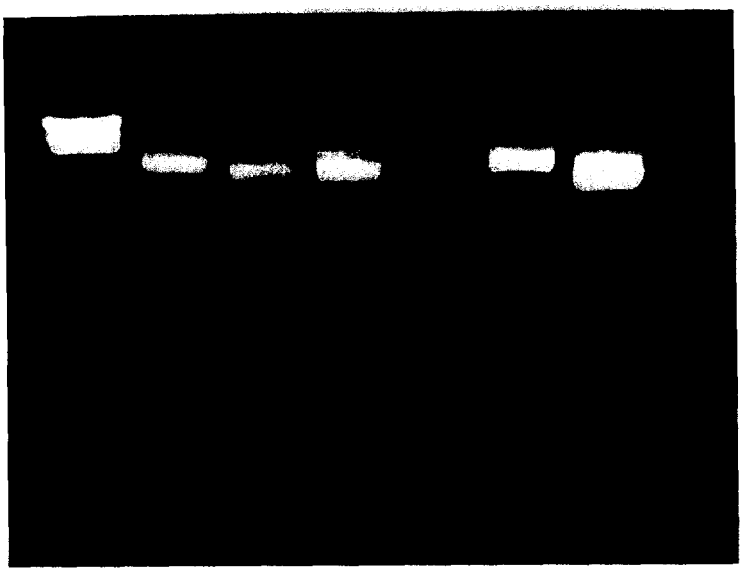

Figure 8. A gel electrophoresis on plasmid DNA isolated with the aid of Bio-Rad's Quantum Prep Plasmid Miniprep Kit. As can be see, lanes 2 (42A) and 6(18 A), which have not migrated as far as lanes 3, 4, and 6, contain the pT-Adv vector inserts (C. Rigas 1999).

(listed in Table 2). It was hypothesized, however, that there may be the existence of "baby inserts", i.e., inserts smaller than normal. Thus, this percentage may actually be higher. In applying this percentage to the total sorted library, however, approximately 115 of the 208 colonies stored should show detectable inserts.

Table 2. Samples screened

Plates	Samples with Inserts	Samples with no inserts
A	13A, 6A, 3A, 24A, 42 A, 4A, 18A, 9A,1A, 21A, 22A, 34A,41A	12 A, 29A, 38A, 17A, 40A, 19A,
B		24B, 22B
E	44E, 12E, 51E, 43E, 48E, 42E, 43E	20E, 28E, 48E, 7E, 2E, 4E, 41E, 3E.5E,1E,

Table 3. Samples sent for sequencing

Samples sent for Sequencing
24 A, 42A, 9A, 4A, 18A,3A, 6A, 13A

DNA Sequencing

Of the 20 samples shown to contain inserts, 8 were sent to Riley Hospital in Indianapolis to be sequenced by Heather Bullock and Audra Carroll, two past graduate researchers in our lab. Of those 8 sent, Audra and Heather were able to sequence one gene and run a BLAST search to discover whether this rare differentially expressed gene yielded homology to anything that was previously sequenced.

The subsequent BLAST search that was conducted via GENBANK determined that this sequenced gene yielded 48.1% homology to *Homo sapiens* mRNA expressed only in placental villi, and 46.1% homology to *Homo sapiens* actin-binding double-zinc-finger protein mRNA and *Homo sapiens* Toll/interleukin-1 receptor mRNA. These results are particularly interesting because they link plant defense genes to genes that are being expressed in humans during developmental, transcriptional, and defense processes. In addition, it has been already established that the *N* gene shows homology to these receptors. Therefore, the sequenced gene isolated from the cDNA subtraction library is most likely an *N* gene, indicating that orchids exhibit plant defense responses mediated by this important *R* gene. As a result, this may be a potential focus of this project in the future.

RNA Isolations

Six RNA isolations were conducted on tissue isolated from the opposite leaf from where the inoculation was made in the infected orchid. The time points in which infected tissue was collected were Day 1, Day 2, Day 11, Day 42, March 29,1999 tissue, and Control tissue. These samples each ranged in weight from 0.200g to 0.338 g. The RNA from each sample was isolated and then read via a spectrophometer to determine yield. The results are listed in Table 3.

Table 3. The Spectrophotometer Readings of RNA isolated from the Opposite Leaf of the Infected Orchid.

DAY OF TISSUE	WEIGHT USED	SPECTROPHOTOMETER READING (260NM)	RNA ISOLATED MICROGRAMS PER MILLILITER	MICROGRAMS OF RNA ISOLATED
Control	0.238 g	0.064	51.2	2.56
Day 1	0.242 g	0.048	38.4	1.92
Day 2	0.325 g	0.064	51.2	2.56
Day 11	0.338 g	0.142	113.6	5.68
Day 42	0.204 g	0.043	34.4	1.72
Now (3/29/99)	0.254 g	0.045	36	1.8

1 Optical Density (O.D.) = 800 μg/mL of RNA.

CONCLUSION

While the hypersensitive response is an area of intense study for molecular botanists, this has not been a hot topic in orchids. Nonetheless, the study of this response in orchids could yield tremendous benefits because of the existence of the Wheeler Orchid Collection. As already discussed, the Odontoglossum Ringspot Virus is very contagious and can be very harmful not just to the infected orchid itself, but also to those located within close proximity. Since the Wheeler Orchid Greenhouse is not large in size, this could be a gigantic problem. Therefore, the study of the HR in orchids will not only provide more information to their defense responses, but also provide methods with which to produce transgenic orchids that are resistant to ORSV.

A particularly interesting result of this research is that the gene sequenced shows 46.1% homology to the Toll/Interleukin-1 receptor mRNA. This indicates that perhaps the gene sequenced is the *N* resistance gene. In the future, a complete sequence of this gene may be obtained and compared to N gene sequences from other plant species.

Currently, because it was determined that rare viral defense genes have been isolated, RNA isolations of the original infected tissue and its opposite leaf collected at different time points throughout the infection are being conducted. Once all the RNA is isolated and yields prove to be sufficient, a Northern Blot will be performed in order to determine when some of genes are "turned on" in the plant defense cycle and how long these genes are expressed after infection. Once this blot analysis is completed, an intensive literature review will be made and probes will be designed via DNASTAR to pick out PR genes, the N-gene, and perhaps those involved in programmed cell death.

Overall, the identification and characterization of these virally induced genes should provide more information about the timing and process of active orchid defense. This, in turn, could lead to new strategies and perhaps technologies with which resistance can incorporated into the plant. Thus, this only the beginning of a new horizon in orchid defense and resistance.

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